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## Myeloid-Related Protein-8/14 and the Risk of Cardiovascular Death or Myocardial Infarction after an Acute Coronary Syndrome in the PROVE IT-TIMI 22 Trial

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### Abstract

**Background**—Using a transcriptional profiling approach, we recently identified myeloid-related protein-8/14 (MRP-8/14) to be expressed by platelets during acute MI. Elevated concentrations of MRP-8/14 are associated with a higher risk for future cardiovascular events in apparently healthy individuals, but have not been assessed with respect to prognosis in patients with ACS.

**Methods**—We performed a nested case-control study (n=237 case-control pairs) among patients enrolled in the PROVE IT-TIMI 22 trial (mean follow-up 24 months) in order to investigate the risk of cardiovascular death or myocardial infarction (MI) associated with MRP-8/14 measured at 30 days after an acute coronary syndrome (ACS).

**Results**—Patients with cardiovascular death or MI after 30 days (cases) had higher median [25<sup>th</sup>, 75<sup>th</sup> percentile] MRP-8/14 levels than patients who remained free of recurrent events (5.6 [2.8, 13.5] mg/L vs 4.0 [1.9, 10.1] mg/L, p = 0.020). The risk of a recurrent cardiovascular event increased with each increasing quartile of MRP-8/14 (P-trend=0.007) such that patients with the highest levels had a 2.0-fold increased odds (95% CI 1.1 – 3.6, p = 0.029) of a recurrent event after adjusting for standard

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risk indicators, randomized treatment, and C-reactive protein. Patients with elevated levels of MRP-8/14 and hsCRP showed significantly increased risk of cardiovascular death or MI compared with patients with the lowest levels of both markers (adj OR 2.0; 95% CI 1.1 – 3.6).

**Conclusions**—MRP-8/14 may be a useful biomarker of platelet and inflammatory disease activity in atherothrombosis and may serve as a novel target for therapeutic intervention.

### Keywords

acute coronary syndromes; biomarkers; risk factors; myocardial infarction

## INTRODUCTION

In patients with acute coronary syndromes (ACS), recurrent cardiovascular events remain a significant medical problem. Among patients managed with aggressive secondary preventive interventions in the intensive statin therapy arm of the PROVE IT-TIMI 22 trial the rate of death, myocardial infarction, unstable angina, need for revascularization or stroke was approximately 22% over a 2-year period.<sup>1</sup> Enhancements to our present tools for risk stratification, especially those that may guide selection of optimal treatment regimens and/or the development of new therapies, are of great interest.<sup>2</sup> Novel biomarker development strategies that originate from characterization of molecular and cellular changes during acute atherothrombosis have particular potential to reveal new targets for both risk stratification and therapy.<sup>3</sup>

Guided by the central role of the platelet in ACS, we previously profiled platelet messenger RNA (mRNA) from patients with acute ST-segment myocardial infarction (MI) or stable coronary artery disease.<sup>4</sup> Since platelets lack a nucleus, the platelet mRNA, called the transcriptome, provides a profile that generally reflects gene expression preceding a specific biologic event (platelets circulate for 7–10 days), without the confounding possibility that the acute event itself has provoked new gene transcription. Using this novel approach, we found that one of the strongest discriminators of MI compared with stable disease was myeloid-related protein-14 (MRP-14, also known as S100A9, calgranulin B).<sup>4</sup> MRP-14 is a member of a family of proteins that have intracellular and extracellular roles modulating calcium signaling, arachadonic acid metabolism, cytoskeletal reorganization, and trafficking of neutrophils.<sup>5</sup> In humans, the most abundant form of MRP-14 is MRP-8/14, in which MRP-14 is bound to MRP-8.<sup>6</sup> Although MRP-8/14 is highly expressed in neutrophils, our recent data indicate that platelets and megakaryocytes also contain MRP-14 mRNA and that platelets express MRP-8/14 protein. We have recently validated the prognostic relevance of the MRP-14 gene target with respect to the risk of first cardiovascular events (nonfatal myocardial infarction or stroke, or cardiovascular death) among apparently healthy post-menopausal women followed in the Women's Health Study.<sup>4</sup>

The present study of patients with ACS enrolled in the PROVE-IT TIMI 22 Trial was designed to test the hypothesis that elevated plasma levels of MRP-8/14 may identify patients with ACS at heightened risk for recurrent cardiac events.

## METHODS

### Study Population

A nested case-control study was conducted among patients randomized in the PROVE-IT TIMI 22 trial. The design and results of PROVE-IT TIMI 22 have been reported previously.<sup>1</sup> In brief, PROVE-IT TIMI 22 was a multi-center, randomized, double-blind trial that evaluated intensive (atorvastatin 80 mg daily) versus moderate (pravastatin 40 mg daily) statin therapy

for the prevention of major adverse cardiac events in 4162 patients stabilized after ACS. Randomization occurred in the first 10 days after presentation with MI or high risk unstable angina (median 7 days). Patients were followed for 18 to 36 months after randomization (mean 24 months). Endpoints were adjudicated by an independent clinical events committee.

We designed a prospective, nested case-control study in which patients with CV death or MI after 30 days (cases, n=237) were matched in a 1:1 ratio with patients who remained free of recurrent cardiovascular events (controls, n=237). Cases and controls were matched on age (within one year), sex, and smoking status (former smoker, current smoker, or nonsmoker).

### Blood sampling

As part of the study protocol, a sample of venous blood was obtained in tubes containing EDTA from the subjects at protocol-defined time-points. Plasma samples were stored at  $-80^{\circ}\text{C}$  or colder. Based upon results with our previous analysis of C-reactive protein, for this analysis, we examined the concentration of MRP-8/14 at Day 30 follow-up after the residual inflammatory influence of the qualifying ischemic event would have likely resolved. Case and control specimens were assayed for high-sensitivity CRP and lipids, as previously described.<sup>7</sup> Plasma MRP-8/14 levels were measured by ELISA (Bühlmann Laboratories, Schönenbuch, Switzerland). Performance characteristics of this assay include intra-assay imprecision of 4.8% at 3.4 mg/L, inter-assay imprecision of 4.4% at 5.1 mg/L, minimal detectable concentration of 0.3 mg/L, and functional sensitivity (level of 15% imprecision) of 0.56 mg/L. When examined in apparently healthy women, the median plasma concentration was 2.1 mg/L (25<sup>th</sup>, 75<sup>th</sup> percentiles: 1.2, 3.4 mg/L).<sup>4</sup> Data for the monocyte-derived marker neopterin were also available in 76 patients. All biomarker testing was performed by personnel who were blinded to treatment arms, outcomes, and results of other laboratory testing.

### Statistical analysis

The plasma concentrations of MRP-8/14 are reported as the median (25<sup>th</sup>, 75<sup>th</sup> percentiles). Baseline characteristics treated as continuous variables were compared with the Wilcoxon signed rank test for paired data, and categorical variables were compared with McNemar's test. Given a non-parametric distribution of MRP-8/14, concentrations of the marker were compared using the Wilcoxon signed rank test for paired data. Correlations between levels of MRP-8/14, lipids, and CRP were examined with Spearman correlation coefficient. To evaluate its association with CV death or MI, MRP-8/14 was analyzed categorized into quartiles according to the concentration MRP-8/14 at 30 days. A landmark analysis was performed using conditional logistic regression from the time of sampling (treating 30 days as time zero for the analysis of subsequent events) and including all patients with an available MRP-8/14 result. Confirmatory analyses excluding patients with non-fatal ischemic events prior to the 30 day visit were also performed to eliminate possible confounding by recent cardiac events resulting in an increase in MRP-8/14 at 30 days. Tests for linear trends were computed using an ordinal variable for biomarker quartiles. Standard logistic regression was used for analyses within unmatched subgroups. Adjusted risk estimates were obtained from regression models that, in addition to accounting for matching (age, sex, smoking status), adjusted for the qualifying syndrome (ST-elevation MI, non-ST elevation MI or unstable angina), history of diabetes, history of HTN, prior coronary artery disease, prior peripheral arterial disease, prior cerebrovascular disease, prior heart failure, aspirin at discharge, achieved LDL levels, other biomarkers including hsCRP, and randomized treatment.

All analyses were performed using STATA v9.2 (STATA Corp., College Station, Texas). All P-values were two-tailed, and values of less than 0.05 were considered to indicate statistical significance. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

## RESULTS

We performed a nested case-control study (n=237 case-control pairs) among patients enrolled in the PROVE IT-TIMI 22 trial to assess the risk of cardiovascular death or MI (mean follow-up 24 months), associated with plasma levels of MRP-8/14 measured 30 days after ACS. Patients with CV death or MI after 30 days (cases) were matched in a 1:1 ratio with patients who remained free of recurrent cardiovascular events during study follow-up (controls). The baseline characteristics of the 237 cases and 237 control subjects are shown in Table 1. As anticipated, cases (including 41 cardiovascular deaths) had a higher frequency of cardiovascular risk factors than controls. Because of matching, age, sex, and smoking status were virtually identical between study groups. The observed range of MRP-8/14 in this study was 0.3 to 101 mg/L with a median concentration of 4.9 mg/L. Women ( $p = 0.046$ ), and patients with a history of diabetes ( $p = 0.009$ ) and hypertension ( $p = 0.006$ ) were more likely to have a concentration of MRP-8/14 above the median (Table 1b Online only). The concentration of MRP-8/14 was not significantly associated with age, self-reported race, smoking status, or the qualifying event (STEMI, NSTEMI, or UA), ( $p > 0.05$  for each). Spearman correlation coefficients between MRP-8/14 and hsCRP, white blood cell count, neopterin (a monocyte-derived marker), LDL, and HDL were 0.25, 0.14, 0.17, 0.045, and 0.060, respectively.

The concentration of MRP-8/14 at 30 days was significantly higher in patients with CV death or new MI during subsequent follow-up (cases) than in patients who remained free of recurrent events (controls) (Table 2,  $P = 0.020$ ). In matched-pair analysis accounting for age, sex and smoking status, the relative odds of CV death or MI increased significantly with each increasing quartile of baseline concentration of MRP-8/14 (Table 3,  $P$ -trend = 0.007) such that patients in the highest versus lowest quartile had a 2-fold elevation in risk (OR 2.0; 95% CI 1.2 – 3.4;  $P = 0.009$ ). The relative risk associated with MRP-8/14 for important subgroups, including those allocated to intensive statin therapy, are shown in Figure 1. There was no significant heterogeneity of the risk associated with MRP-8/14 among these subgroups.

After adjustment for unmatched clinical risk indicators (qualifying syndrome, history of diabetes, history of hypertension, prior coronary artery, peripheral arterial or cerebrovascular disease, heart failure, aspirin at discharge, achieved LDL levels), and randomized treatment allocation, the concentration of MRP-8/14 remained associated with the risk of CV death or MI (Table 3,  $P$ -trend=0.017). When additionally adjusted for hs-CRP, patients in the highest quartile of MRP-8/14 had a 2.0-fold higher odds of CV death or MI (95% CI, 1.1 – 3.6,  $p = 0.029$ ). This relationship persisted when patients with any non-fatal recurrent ischemic event between enrollment and Day 30 were excluded (adjusted OR 2.0; 95% CI 1.1 – 3.8,  $p = 0.033$ ). The combined use of MRP-8/14 and hsCRP for risk stratification is illustrated in Figure 2, showing an additive relationship with the risk of CV death or MI. When treated as a continuous variable (log-transformed), each natural log increase in MRP-8/14 was associated with a 20% increase in the risk of CV death or MI ( $p = 0.049$ ), after adjusting for clinical risk indicators, and biomarkers, including hsCRP. In an additional analysis limited to those case-control pairs with measurement of lipoprotein phospholipase A2 activity (N=396), the risk associated with the highest quartile of MRP-8/14 remained significant after adding this additional biomarker to the model (adjusted OR 2.4; 95% CI 1.2 – 5.0,  $p = 0.014$ ). Moreover, after further adjusting for B-type natriuretic peptide (N=314), the risk associated with the highest quartile of MRP-8/14 remained significant (adjusted OR 2.5; 95% CI 1.1 – 5.7,  $p = 0.025$ ).

The concentration of MRP-8/14 at 30 days were lower in patients treated with atorvastatin 80 mg daily compared with pravastatin 40 mg daily (4.0 mg/L vs. 5.6 mg/L,  $p = 0.046$ ). There was no detectable modification of the risk associated with MRP-8/14 based upon treatment allocation ( $p$ -interaction = 0.84).

## DISCUSSION

This study demonstrates that in a cohort of patients with ACS, the plasma concentration of MRP-8/14 is independently associated with the risk for recurrent cardiovascular events. Notably, this protein expressed by both platelets and inflammatory cells was only weakly correlated with hsCRP and monocyte-derived neopterin. Importantly, the association with outcome was independent of traditional clinical risk indicators and hsCRP. These findings provide valuable proof-in-principle for the platelet transcriptional profiling strategy leading to the evaluation of MRP-8/14 in cardiovascular disease. The results also support the possible relevance of platelets as participants in inflammatory processes underlying acute atherothrombosis, and point toward a potential new therapeutic target.

### MRP-8/14 as a Novel Biomarker

Platelets and neutrophils play important pathophysiologic roles in ACS,<sup>8, 9</sup> and the measurement of biomarkers related to their activity provides independent prognostic information.<sup>10, 11</sup> Like soluble CD40L,<sup>12</sup> MRP-8/14 may reflect the interrelated activation of platelets and inflammatory cells contributing to an environment that characterizes vulnerable plaque and perhaps the “vulnerable” patient.<sup>13</sup> Although plasma MRP-8/14 was previously considered leukocyte-derived,<sup>6</sup> our recent study raises the possibility that platelets and megakaryocytes may serve as an additional source of MRP-8/14.<sup>4</sup> Because of its ability to regulate calcium signaling and promote cellular cytoskeletal reorganization,<sup>14</sup> it is intriguing to speculate that MRP-14 may be involved in the cellular events that also promote platelet-mediated thrombosis. Nevertheless, additional experimental and clinical studies, including interrogation of platelet mRNA in additional patient populations and evaluation of other soluble biomarkers originating from neutrophils, are likely to be valuable in discerning the relative contribution of platelets and inflammatory cells to the plasma concentration of MRP-8/14.

The serum concentration of MRP-8/14 is a useful biomarker of disease activity in inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel disease.<sup>15</sup> Neutrophils and monocytes highly express MRP-8/14.<sup>6</sup> Inflammatory stimuli promote the surface expression and secretion of MRP-8/14, where it functions, in part, as a chemoattractant regulating leukocyte adhesion.<sup>16, 17</sup> Patients with diabetes mellitus have elevated plasma levels of MRP-8/14,<sup>18</sup> and related members of the same family of proteins bind to receptor for advanced glycation end-products (RAGE) and trigger pro-inflammatory and pro-thrombotic responses.<sup>19</sup> Thus, there are plausible potential pathophysiologic contributions from MRP-8/14 originating either from platelets or leukocytes.

### Clinical and Scientific Implications

The present results have several implications. First, the findings support hypotheses linking inflammation, platelet activation, and thrombosis in the pathogenesis of ACS.<sup>12</sup> There is growing evidence that neutrophils play an important pathophysiologic role in ACS.<sup>10, 20</sup> MRP-8/14 is the most abundant cytosolic protein in neutrophils and is essential for the recruitment of neutrophils during wound healing *in vivo*.<sup>14</sup> Thus, it is possible that MRP-8/14 may not only be a marker of neutrophil activation, but also may be a direct contributor to inflammatory and thrombotic responses during ACS. Indeed, observations in MRP-14-deficient mice indicate that MRP-14 is essential for the recruitment of neutrophils at sites of injury.<sup>14</sup>

Second, the data indicate that MRP-8/14 may add prognostic information to that conveyed by standard risk factors and CRP in patients with recent ACS. This finding extends our observation in otherwise healthy women at risk for first cardiovascular events among whom we observed a 3.8-fold increase risk in any vascular event ( $P<0.001$ ) among those with the highest levels



of MRP-8/14. Additional investigation is needed to refine quantitative estimates of the risk relationships with MRP-8/14 in this and other populations and to define its value as a potential contributor to multi-marker strategies for risk assessment.<sup>2</sup> It is noteworthy that consistent with prior observations regarding potential pleiotropic effects of statins,<sup>21</sup> that patients treated with the intensive statin strategy tended toward lower levels of MRP-8/14 by 30 days. This finding was of borderline statistical significance and warrants corroboration in additional separate studies.

Third, these results provide an encouraging example of success in meeting the benchmark of external clinical validation of a marker originating from a genomic-based strategy for novel biomarker development.

## Limitations

Several limitations of the study merit consideration. First, our study design does not permit us to determine the precise cellular source of plasma MRP-8/14. The relative contributions of platelets and leukocytes to the plasma pool of MRP-8/14 are the focus of ongoing studies. Second, platelets possess a complex that processes pre-mRNA in the “mature” mRNA that is translated into protein.<sup>22</sup> Thus, it is possible that changes in mRNA processing may modulate the platelet transcriptome (including mRNA for MRP-14) in response to activation during a clinical event such as MI. Third, owing to the timing of blood sampling in this study, our results can not be generalized to measurement of MRP-8/14 at the time of initial presentation. Fourth, our case-control design does not permit us to fully evaluate the interaction of statin therapy with the risk associated with the achieved levels of LDL cholesterol and MRP-8/14 during statin therapy. Finally, data on recovery of MRP-8/14 after long-term storage are not available. However, any variability introduced by loss of recovery would be expected to have drawn our findings toward the null hypothesis.

## Conclusion

In conclusion, we found that MRP-8/14 is independently associated with the risk of recurrent cardiovascular events in patients with ACS. These data indicate that MRP-8/14 may be a useful biomarker of platelet and inflammatory disease activity in atherothrombosis and may serve as an interesting target to explore for therapeutic intervention.

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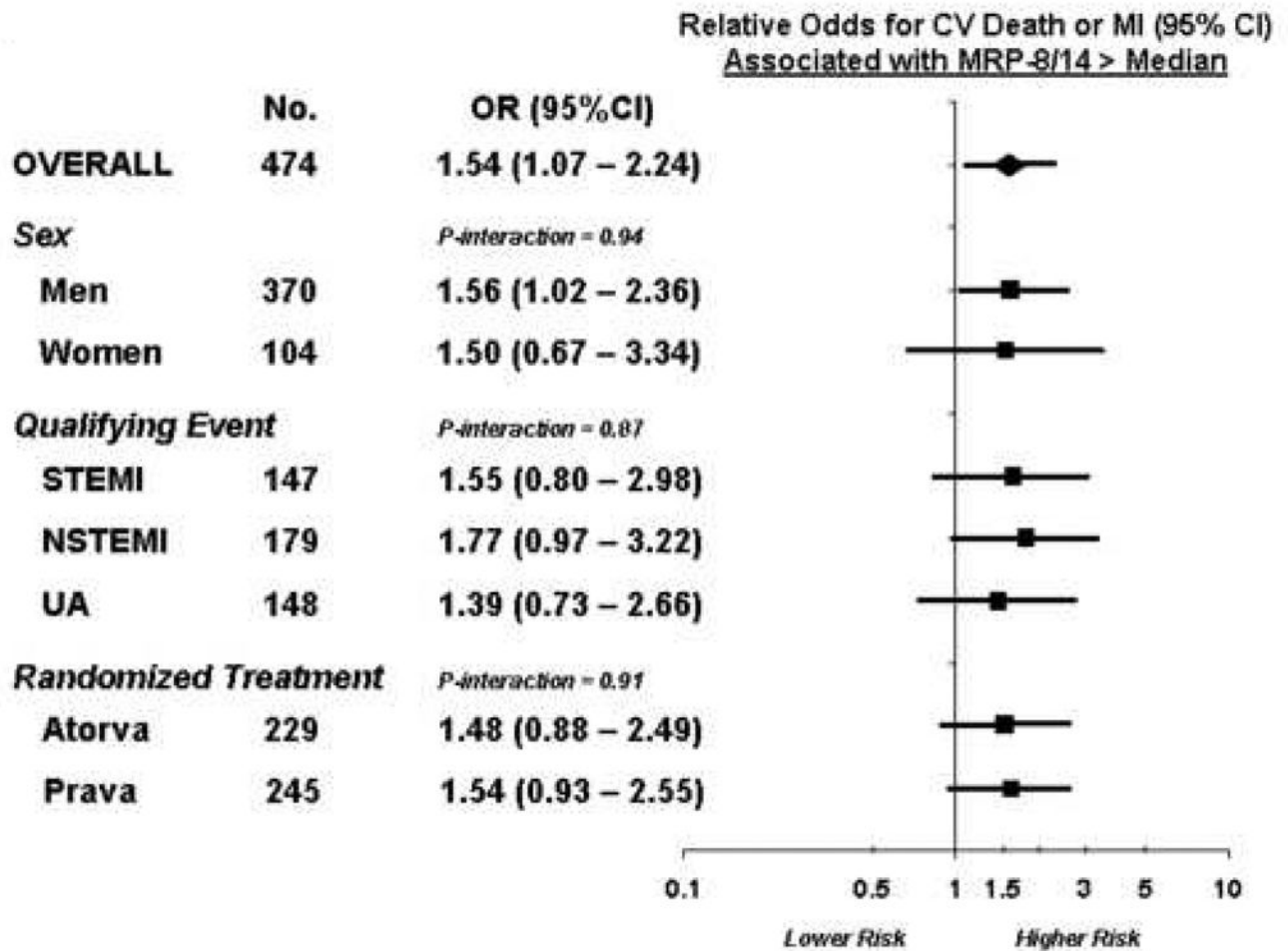
DIME, and NCME. Dr. Braunwald has received honoraria from Bristol Myers Squibb, Merck and Pfizer, and has served as a consultant to Bristol-Myers Squibb, and Pfizer. Dr. Simon serves on advisory boards for Cordis/Johnson & Johnson, Millennium, and Schering-Plough, receives grant support from Accumetrics, Cordis/Johnson & Johnson, Millennium, and Schering-Plough, and has received lecture fees or honoraria from Accumetrics, Boston Scientific, Cordis/Johnson & Johnson, Millennium, Sanofi-Aventis, and Schering-Plough. Drs Wang, Croce, Sakuma, Sabatine, Gao, and Healy, and Ms Buross and McCabe have no additional relationships to report.

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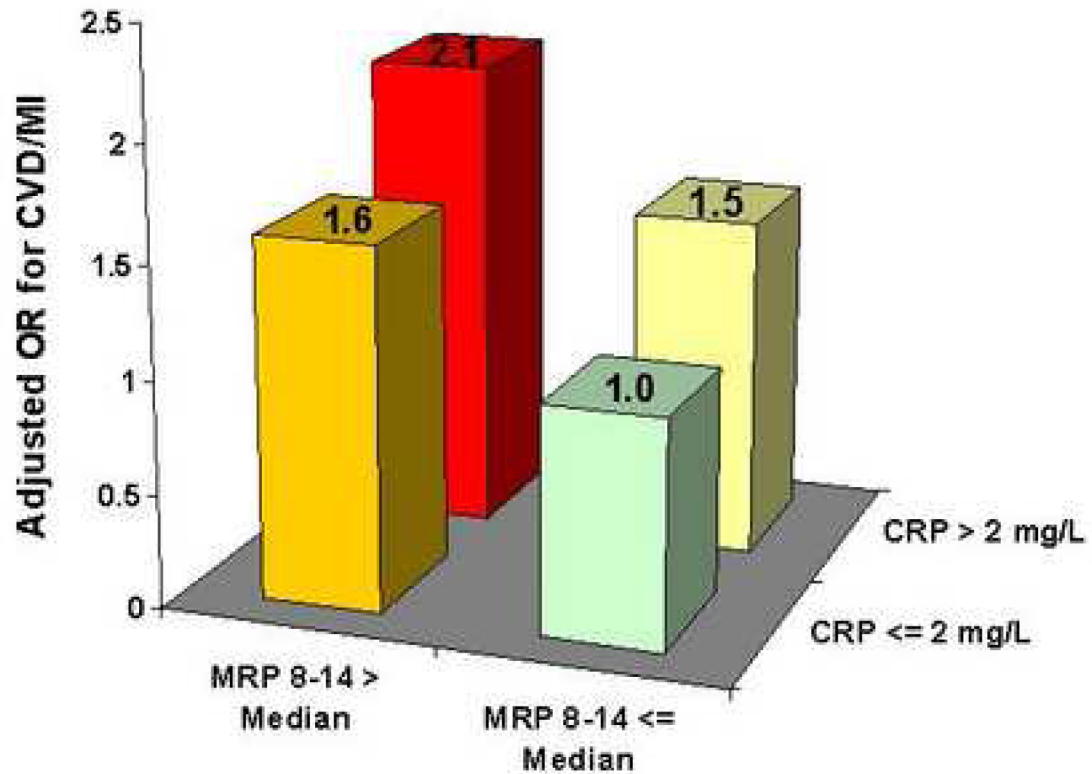
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**Figure 1.**  
Relative odds of CV death or MI associated with MRP-8/14 concentration above the median stratified by sex, qualifying syndrome, and randomized treatment.



**Figure 2.**

Adjusted relative odds of CV death or MI according to MRP-8/14 and hsCRP. Patients with elevated MRP-8/14 and hsCRP were at 2.1 fold (95% CI 1.2 – 3.8) higher risk of CV death or MI compared to those with a low plasma concentration of both biomarkers, after adjusting for qualifying syndrome, history of diabetes, history of hypertension, prior MI, heart failure, aspirin at discharge, achieved LDL levels, and randomized treatment allocation. There were 128, 108, 97 and 140 patients in the low MRP-8/14/low hsCRP, low MRP-8/14/high hsCRP, high MRP-8/14/low hsCRP, and high MRP-8/14/high hsCRP groups respectively.

**Table 1**  
Characteristics of the Nested Case-Control Population

		Controls (n = 237)	Cases (n = 237)	p value
<b>Demographics</b>				
Age, yrs		61 (52, 70)	61 (52, 70)	-
Female		52 (21.9%)	52 (21.9%)	-
<b>Risk Factors</b>				
Current smoker		95 (40.1)	95 (40.1)	-
Hypertension		110 (46.4)	140 (59.1)	0.007
Diabetes		45 (19.0)	67 (28.3)	0.017
BMI (kg/m <sup>2</sup> )		28.1 (25.7, 30.6)	28.7 (25.8, 32.1)	0.06
<b>Cardiovascular History</b>				
Coronary artery disease		96 (40.5)	125 (52.7)	0.008
Cerebrovascular disease		13 (5.5)	28 (11.8)	0.011
Peripheral arterial disease		15 (6.3)	40 (16.9)	0.0003
<b>Presenting Characteristics</b>				
Qualifying event	STEMI	64 (27.0)	83 (35.0)	0.060
	NSTEMI	101 (42.6)	78 (32.9)	
	UA	72 (30.4)	76 (32.1)	
Heart Failure		9 (3.8)	19 (8.1)	0.080
<b>Concomitant therapies at discharge</b>				
Aspirin		192 (81.0)	170 (71.7)	0.021
Thienopyridine		142 (59.9)	145 (61.2)	0.84
Atorvastatin 80 mg		117 (49.4)	109 (46.0)	0.52
ACE inhibitors		117 (49.4)	131 (55.3)	0.22

Data are shown as N (%) for dichotomous variables and median (25<sup>th</sup>, 75<sup>th</sup> percentile) for continuous variables. STEMI = ST-elevation myocardial infarction; NSTEMI = non-ST elevation myocardial infarction; UA = unstable angina; ACE = angiotensin converting enzyme

**Table 2****Biomarker Concentrations in Case and Controls**

	<b>Controls</b>	<b>Cases</b>	<b>p value</b>
MRP-8/18 (mg/L)	4.0 (1.9 – 10.2)	5.6 (2.8 – 13.5)	0.020
hs-CRP (g/L)	1.9 (0.8, 3.8)	2.5 (1.2, 5.8)	0.0006
Neopterin *	8.6 (7.4, 12.2)	8.9 (6.6, 11.7)	0.17
LpPLA2 activity	32.9 (25.9, 42.5)	36.5 (28.3, 47.5)	0.050
LDL-C (mg/dL)	74 (57, 98)	79 (57, 98)	0.17
HDL-C (mg/dL)	40 (33, 47)	41 (34, 48)	0.70
Triglycerides (mg/dL)	120 (85, 152)	123 (93, 186)	0.004
eGFR	76.8 (63, 90)	73.5 (63, 88)	0.16

Data are shown as median (25<sup>th</sup>, 75<sup>th</sup> percentile).

\*  
N = 76

**Table 3**  
Relative Odds of Cardiovascular Death or MI According to MRP-8/14 Concentration

		Quartile of MRP-8/14 Concentration (Range, mg/L)				P-trend
		Q1 (<21)	Q2 (21 - <49)	Q3 (49 - 110)	Q4 (>110)	
Crude matched pairs	OR	1.0	1.3	1.6	2.0	0.007
	(95% CI)	-	0.79 - 2.3	0.95 - 2.7	1.2 - 3.4	
	P	-	0.29	0.077	0.009	
Adj for clinical variables <sup>†</sup>	OR	1.0	1.4	1.8	2.0	0.017
	(95% CI)	-	0.77 - 2.6	0.97 - 3.2	1.1 - 3.7	
	P	-	0.26	0.06	0.020	
Additionally adj for hsCRP	OR	1.0	1.3	1.7	2.0	0.024
	(95% CI)	-	0.73 - 2.5	0.91 - 3.1	1.1 - 3.6	
	P	-	0.34	0.099	0.029	

<sup>†</sup> Adjusted for the qualifying syndrome, history of diabetes, history of hypertension, prior coronary artery disease, prior cerebrovascular disease, prior peripheral vascular disease, prior heart failure, aspirin at discharge, achieved LDL levels, and randomized treatment allocation.